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Abstract: Research on plant responses to temperature stress is receiving increased interest due to the growing awareness about global warming. High and low temperature stresses help establish the narrow geographic distribution of some cultivated plants, the limited geographic extension of some other economically nutritionally important species, and also induce irregular bearing for some species. However, the understanding of plant responses to temperature stress lags behind other biotic and abiotic stresses probably due to the complex response at the molecular, cellular, and organismal level. Temperature stress affects, indeed, many developmental processes during the plant's life cycle. However, the reproductive stage, the outcome of which represents the economic value for many cultivated plants, is especially vulnerable. Here the effect of low and high temperature stresses during the flowering phase is reviewed in flowering plants in an attempt to unravel sensitive stages that are behind irregular cropping. The review presents detailed findings from 33 previously published reports spanning 19 different flowering plant species. Both the male and female organs of the flower are especially sensitive to temperature fluctuations both during their development before pollination and during the post-pollination stage. The effect of temperature stress is, however, obscured by the complex male–female interaction superimposed on the individual behavior of each organ. Interestingly, a review of the literature on this topic shows that genetic variation does exist in reproductive behavior under temperature fluctuations. This genetic diversity must be preserved and characterized in further detail to understand how plants naturally cope with changing environmental conditions, which will, undoubtedly, help us to design better strategies to face current and future challenging temperature fluctuations.

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Sensitivity of flowering plant gametophytes to temperature fluctuations

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ABSTRACT

Research on plant responses to temperature stress is receiving increased interest due to the growing awareness about global warming. High and low temperature stresses help establish the narrow geographic distribution of some cultivated plants, the limited geographic extension of some other economically-nutritionally important species, and also induce irregular bearing for some species. However, the understanding of plant responses to temperature stress lags behind other biotic and abiotic stresses probably due to the complex response at the molecular, cellular, and organismal level. Temperature stress affects, indeed, many developmental processes during the plant's life cycle. However, the reproductive stage, the outcome of which represents the economic value for many cultivated plants, is especially vulnerable. Here the effect of low and high temperature stresses during the flowering phase is reviewed in flowering plants in an attempt to unravel sensitive stages that are behind irregular cropping. The review presents detailed findings from 33 previously published reports spanning 19 different flowering plant species. Both the male and female organs of the flower are especially sensitive to temperature fluctuations both during their development before pollination and during the post-pollination stage. The effect of temperature stress is, however, obscured by the complex male-female interaction superimposed on the individual behavior of each organ. Interestingly, a review of the literature on this topic shows that genetic variation does exist in reproductive behavior under temperature fluctuations. This genetic diversity must be preserved and characterized in further detail to understand how plants naturally cope with changing environmental conditions, which will, undoubtedly, help us to design better strategies to face current and future challenging temperature fluctuations.

KEYWORDS:

Heat stress, cold stress, crop yield, flowering plants, male gametophyte, female gametophyte.

Content

1. Introduction

Temperature stress affects different plant developmental and physiological processes (Hall 2001) and is considered one of the major constrains to plant adaptation, especially when it coincides with critical stages of plant development (McWilliam, 1980). One of the main objectives in plant domestication has been to spread cultivated plants along a larger geographical range and significant efforts have been dedicated to increase plant tolerance/avoidance to temperature fluctuations (Hall, 2001). Furthermore, studies on temperature tolerance in different crops are needed, now more than ever before, for two major reasons. First, although technological progress during the last century has pushed yields close to their maximum potential in many species, it has barely kept pace with population growth, which is expected to raise up by 2 to 4 billions by 2050 (Cohen, 2003; Trewavas, 2002). However, increasing available farmlands is hardly feasible without plowing up virgin lands and forests and, thus, deteriorating further the already threatened natural habitats and biodiversity (Trewavas, 2002). Second, the recent linear trend of 0.74ºC increase in the average global near-surface temperature registered between 1906 and 2005 (Solomon et al., 2007) seems to support the fact that, indeed, we are facing an unequivocal global warming which may represent another challenge to plant productivity and geographic distribution (Hedhly et al., 2009).

For many cultivated plant species the economic value lies in the outcome of the sexual phase, resulting in the seed and/or the fruit. It is known that temperature variation outside the range of species tolerance during flowering can jeopardize fruit and seed set. Both in cultivated and wild plant species low and high temperatures have been shown to make fruit set erratic if not null (section 2). The reported underlying causes of such effects are, however, very diverse. This is because, first, both the timing and extension of temperature stress –either natural or experimental- are remarkably variable. Second, because species, and even varieties/landraces/ecotypes within species, differ in their sensitivity to temperature and/or in the particular stage that is the most sensitive.

This review examines the implications of temperature stress during the flowering phase on fruit and seed set in cultivated and wild plant species. It shows that (i) both low and high temperature stresses during the flowering phase could largely explain erratic and reduced fruit and seed set; (ii) the three genetically different elements that are present and interact within a flower, the sporophyte -pistil tissues- and the two gametophytes- pollen and embryo sac, are specially sensitive to temperature stress; and (iii) genetic variation that likely reflect adaptation to temperature stress does exist at both the interspecific and intraspecific levels.

2. Low and high temperature stresses at flowering result in erratic fruit/seed set

Many cultivated and wild plant species within different climatic regions worldwide perform poorly and display erratic fruit and seed set when subjected during their flowering phase to high and low temperatures beyond their optimum needs. For example, in temperate fruit trees, a substantial reduction in fruit set has been shown in apricot (*Prunus armeniaca* L.) after an increase in the average daily temperature as low as 3ºC during the week preceding anthesis (Rodrigo and Herrero, 2002), or in sweet cherry (*Prunus avium* L.) during the first two weeks following anthesis (Hedhly et al., 2007). Similarly, results in peach (*Prunus persica* L. Batsch), show that a two month exposure to different constant temperatures in growth chambers, starting 1 month before anthesis, resulted in very low fruit set above 25ºC (Kozai et al., 2004). In pear (*Pyrus communis* L.), Mellenthin et al. (1972) found a linear relationship between mean maximum temperatures registered during the first 10 days following anthesis and fruit set. Likewise, tropical fruit trees are usually sensitive, during their flowering phase, to temperature fluctuations in subtropical and temperate regions, a characteristic that limits the extension of their cultivation range. Mango (*Mangifera indica* L.) has been shown to be prone to recurrently erratic fruit set with an increased proportion of stenocarpic fruits when temperature falls below 10ºC during flowering (Sukhvibul et al., 2005). Cherimoya (*Annona cherimola* Mill.), native to the tropical highlands of the Andes, usually displays lower fruit set in tropical lowlands and in temperate regions. An increase in temperature from 20ºC to 30ºC during two consecutive years reduced substantially fruit set and increased the number of malformed fruits (Higuchi et al., 1998). In avocado (*Persea americana* L.), higher fruit set has been obtained in the range of 20-25ºC; temperatures outside this range negatively affected fruit set either by impairing fertilization (17ºC) or by abscission of unopened flowers (33ºC) (Sedgley, 1977).

Annual crops also suffer from both low and high temperature stresses occurring during the flowering phase. Due to their economic importance, the effect of temperature stress on the reproductive process has been extensively studied in cereals (see Barnabás et al., 2008 and Thakur et al., 2010 for extensive reviews on the subject). Special mention must be given to the work done in rice (*Oryza sativa* L.), initiated in the 1970s by Japanese researchers (Nishiyama, 1984; Satake and Hayase, 1970) and later confirmed by many authors (e.g. Jagadish et al., 2007; Mamun et al., 2006; Prasad et al., 2006), relating chilling and heat stress during booting (early gametophyte development) and flowering to higher spikelet sterility. Similarly, the effect of high temperatures during flowering in tomato (*Solanum lycopersicum* L.) has also been studied in detail, and not only extreme high temperatures, but even a mild increase in temperature during a short spell coincident with some flowering stages have been shown to affect negatively fruit set

(Abdulbaki and Stommel, 1995; Sato et al., 2002). Bell pepper (*Capsicum annum* L.) has been reported to be equally sensitive to high temperature stress during flowering (Erickson and Markhart, 2002). Rapeseed, flax, groundnut, common bean, cowpea and *Trifolium repens* are some of the many other species, with economic importance in some regions of the world, that are sensitive to temperature fluctuations during flowering resulting in lower fruit and seed set (refer to supplementary table for further details on these and other species).

Although scarcer effort has been dedicated to the effect of temperature stress on seed and fruit set in natural populations, sensitivity to temperature stress during flowering has also been shown for some species (e.g. *Primula* sp.: (Mckee and Richards, 1998); *Plantago lanceolata*: (Lacey and Herr, 2000)). In summary, temperature fluctuations outside the range of species tolerance occurring during the flowering phase seem to alter seed and fruit set in different species and in diverse climatic regions worldwide. In the next 3 sections I will try to answer two main questions, namely; (in section 3) why is flowering so sensitive to temperature stress? and (in section 4 and 5) what are the specific reproductive processes or components that underlie such sensitivity?

3. Flowering stage sensitivity to temperature fluctuations

Temperature stress during flowering affects yield. First, because flowers are the organs that develop into fruits, and any biotic or abiotic pressure that affects their likelihood to set seeds and/or fruits necessarily affects yield. Second, some target oriented works carried out to unveil temperature sensitive stages during plant development have found recurrently that the reproductive process is the most sensitive stage to temperature stress showing a narrow tolerance threshold to temperature fluctuations (Porter and Semenov, 2005). For simplicity, these studies can be categorized into: (i) those applying the stress at different developmental stages, including vegetative growth, and analyzing the effect on both vegetative and reproductive development, and (ii) those confining the stress to some specific stages of reproductive development and searching for the determinant phase; examples from these two approaches are further detailed in supplementary table 1.

Within the first approach, subjecting wheat plants (*Triticum aestivum* L.) to a heat period of eight days during the double-ridge stage (25ºC) and/or during anthesis (35ºC) revealed that grain yield was only significantly reduced when treatments were applied at both stages or at anthesis alone (Wollenweber et al., 2003). Rice subjected to 34ºC (6ºC increase over the control) displayed higher leaf expansion and greater biomass, but grain yield decreased 10% for each 1ºC increase (Baker et al., 1992). In a different series of experiments carried out in common bean (*Phaseolus vulgaris* L.) (Prasad et al., 2002) and groundnut (*Arachis hypogaea* L.) (Prasad et al., 2003), and characterizing the

combined effect of high temperatures and CO2, the beneficial effect of CO2 increase on vegetative growth and photosynthesis was not sufficient to offset the negative effects of high temperature on reproductive processes and yield. In tomato, although moderately elevated temperature stress (METS; 32/26ºC) did not cause significant changes in biomass, photosynthesis, and night respiration, fruit set was significantly reduced (Sato et al., 2000). In some studies of floral abortion under temperature stress, the observed reduction in carbohydrate supply to the reproductive tissues was attributed rather to reduction in sink demand (e.g. abscission of reproductive structures) than to assimilate supply by photosynthetic tissues in tomato (Dinar and Rudich, 1985), pepper (Aloni et al., 1991), and cowpea (*Vigna unguiculata* L.) (Ahmed et al., 1993). Thus, without neglecting the importance of vegetative growth as the source of nutrients and reserves for sink reproductive structures, it seems that small temperature fluctuations -in magnitude and span- that do not necessarily result in great effects on vegetative growth and photosynthetic activity, could be a limiting factor for the likelihood of a flower to set seeds.

Once the sensitivity of reproductive development to temperature fluctuations is known, the second approach to study flower sensitivity to temperature fluctuations is based on differentially restricting the stress to the various stages of the reproductive development, independently or in combination, to study which is the most determinant phase to produce lower fruit and seed set (Figure 1). At the pre-pollination level the effect of temperature has been traditionally focused on the male side, both on the sporophytic tissues of the anther and on the pollen itself. In mild cases, temperature stress leads to a reduction in pollen production and pollen viability, which interferes with post-pollination functioning affecting the fertilization level. On severe cases, temperature stress leads to complete pollen sterility and/or inhibition of anther dehiscence, which can result in no fruit set. Similar effects have been reported on the pistilar sporophytic tissues (stigma, style, and ovary) and on the female gametophyte leading either to poor post-pollination functioning or to female sterility. Temperature stress confined to the post-pollination stage reduces fecundity by affecting both pollen tube growth and pistil and female gametophytic function (e.g. stigmatic receptivity, ovule viability). These individual effects are likely to disturb the largely overlooked pollen-pistil developmental synchrony (Figure 1), which would equally reduce the fertilization level.

Although the particular reproductive critical stage seems to be species-specific and depends on stress intensity and span, the reproductive process as a whole, which usually occurs during a narrow window during plant development, seems to be especially sensitive. For simplicity, we can categorize the reproductive developmental stages more affected by temperature stress as related to the three individuals that are present and interact with each other in the flower: the male, the female gametophytes and the

sporophytic tissues -anther and pistilar tissues-. In the next section I will go through all these reproductive processes highlighting, when they are known, the underlying mechanisms behind the effect of temperature stress.

4. Male development: pollen development and function

Pollen is reported to be sensitive to temperature stress during its whole life span, from the very early developmental stages in the anther up to the double fertilization of the female gametophyte. During the developmental phase before anther dehiscence, temperature fluctuations can affect both proper gametophyte development and the sporophytic anther wall layers. The latter effect would in turn affect gametophyte development (which development is dependent on the tapetum layers of the anther) and/or anther dehiscence, leading to varying degrees of male sterility through proper pollen sterility, reduced pollen production or reduced anther dehiscence. Meiosis has been recurrently reported as a highly sensitive stage to temperature in many species (Ahmed et al., 1992; Clarke and Siddique, 2004; Erickson and Markhart, 2002; Prasad et al., 2003), although the precise mechanism underlying this sensitivity is still not well known. Premature dissolution of the callose wall that surrounds dividing microspores (late meiosis) and the subsequent poor wall development in the resultant microspores has been interpreted as the main reason for pollen sterility in rice under cold stress (Mamun et al., 2006). Early microspore development was reported to be also highly sensitive to chilling stress in the same species (Mamun et al., 2006). Tapetum -the innermost layer of the anther wall- hypertrophy in rice (Nishiyama, 1984) and its earlier degeneration in many other species (Ahmed et al., 1992; Erickson and Markhart, 2002; Porch and Jahn, 2001), which deprives developing pollen grains from essential nutrients and metabolites, is another symptom frequently reported as accompanying pollen sterility under low and high temperature stresses. However, development of the external layers of the anther wall, such as epidermis, endothecium, stomium and septum, has also been shown to be disrupted (Ahmed et al., 1992; Matsui and Omasa, 2002; Porch and Jahn, 2001; Sato et al., 2002), finally affecting anther dehiscence and leading to male sterility even though the pollen is viable. Major alterations in gene expression under high temperature stress have been shown, paralleling tapetum degeneration and pollen sterility, in barley (*Hordeum vulgare* L.) (Abiko et al., 2005) and rice (Endo et al., 2009). Among these genes, enzymes involved in carbohydrate metabolism (e.g. cell wall and vacuolar invertase, sucrose synthase) and transport, are gaining higher research interest as indicators of losses in pollen viability due to temperature fluctuations. Both cold (Oliver et al., 2005) and heat stress (Pressman et al., 2006; Sato et al., 2006) have been shown to down regulate gene expression of several invertase and sucrose synthase isomorphs, and this inhibition was accompanied by a disruption of sucrose and starch

turnover in developing pollen grains, and, hence, lower accumulation of soluble carbohydrates (Aloni et al., 1991; Jain et al., 2007; Oliver et al., 2005; Pressman et al., 2002; Sato et al., 2002). A complete understanding of temperature stress on pollen development must await further understanding of carbohydrate turnover during this phase. Interestingly, in a recent study in barley and Arabidopsis, high temperature stress reduced endogenous synthesis of auxin in developing anthers, an effect that was suggested to be related to pollen sterility since exogenously applied auxin restored fertility (Sakata et al., 2010).

Mature pollen grains, from shedding to arrival to the stigma, appear to be more tolerant to temperature stress than at any other stage of male gametophyte development. Subjecting mature pollen from *Brassica juncea*, *Nicotiana sylvestris* and *Petunia hybrida* to heat shock treatments (60ºC/24h) did not affect their viability and their ability to set fruit (Rao et al., 1992, 1995). Tolerance of pollen grains to low temperature is well documented in studies of pollen storage under ultralow temperatures (Barnabás and Kovács, 1997). Tolerance of mature pollen grains to temperature fluctuations could be attributed to its low plasma water content, to its low metabolic activity, to its protective structures, and to carbohydrate contents and dynamics (Barnabás and Kovács, 1997).

Temperature stress also affects pollen development beyond shedding, from its arrival to the stigmatic surface of the pistil and along its journey toward the female gametophyte within the stylar transmitting tissue and the ovary. Temperature stress affects the very first step in pollen interaction with the pistil, adhesion to the stigma, as well as germination in a number of species (e.g. Hedhly et al., 2005b; Prasad et al., 2006). After penetrating the stigmatic surface, pollen tube growth proceeds along the transmitting tissue of the style and within the ovary towards the female gametophyte. In fact, pollen tube growth rate was the first reproductive characteristic to be evaluated under increasing temperatures, in *Datura stramonium* (Buchholz and Blakeslee, 1927) where pollen tube growth rate increased linearly by a factor of 4.5 from 11ºC to 33ºC (from 1.28 mm/h to 5.86 mm/h). This response pattern of pollen tube growth seems to be a general phenomenon since it has been confirmed in many seed plants, and mathematical models for pollen tube growth in relation to temperature have been developed (e.g. Jefferies et al., 1982). This variation in the rate of pollen tube growth was suggested to be the result of increased metabolism at higher temperatures, typical for most biological growth rates (Lewis, 1942).

5. Female development: the pistil and the female gametophyte

The pistil, the sporophytic maternal tissues that gives shelter and nutritional support to the female gametophyte, also provides nutritional support for the growing male gametophyte. Although traditionally the pistil has been considered as a less sensitive

tissue to temperature stress than the male part (Mascarenhas and Crone, 1996), evidence for pistil sensitivity to changing temperatures is accumulating. Temperature stress affects pre-pollination pistil and embryo sac development as well as their post-pollination function.

A shortening of style length, and abnormalities in ovary development have been reported under mild increases in temperature (an average of 3ºC higher than control) during the last week of flower development in apricot (Rodrigo and Herrero, 2002), as well as under lower than optimal temperatures in mango (20/10ºC, day/night (Sukhvibul et al., 1999)) and chickpea (*Cicer arietinum* L.) (15-20/<8ºC, day/night (Srinivasan et al., 1999)) resulting in lower fruit set. These effects during development were related to failure in post-pollination female function in chickpea (Srinivasan et al., 1999), and in rapeseed (*Brassica napus* L.) (Young et al., 2004). Although temperature stress levels causing negative effects on microsporogenesis did not usually affect macrosporogenesis in many species, abnormalities in embryo sac development have been reported in wheat (30 ºC, (Saini et al., 1983)), and in rapeseed (32/26 ºC, day/night (Polowick and Sawhney, 1988)) reducing seed set in both cases. Moreover, analyzing pollen and pistil function in aborted and retained flowers in chickpea after cold stress, Nayyar et al. (2005) found an even major effect of cold stress on the female function.

The effect of temperature on the sporophytic tissues of the pistil after anthesis has been scarcely documented. This can be explained by the difficulty in separating an effect of temperature on these structures from its effect on pollen germination and tube growth, as well as on pollen-pistil interaction (Figure 1). Thus, for many of the cases in which an effect of temperature has been attributed to failure in pollen adhesion and germination at the stigma or in pollen tube growth within the style (previous section), a parallel failure in stigma and style receptivity cannot be ruled out. The length of stigmatic receptivity is shortened at high temperatures and enlarged at low temperatures in sweet cherry and peach regardless of the effect on the male side (Hedhly et al., 2003, 2005b).

Temperature stress might affect stigma function by affecting the amount of exudates and their temporal availability to pollen grains (Srinivasan et al., 1999). Likewise, the analysis of carbohydrate content in chickpea flowers subjected to cold stress revealed a reduction in carbohydrate levels in aborted styles and ovaries compared to those retained in the plant (Nayyar et al., 2005). Recently, Jain et al. (2007) found a disruption in cell wall invertase activity under low temperature stress not only during the commonly reported pollen developmental phase but also in the stigma and the style. Taken together, these findings point out to a plausible effect of temperature stress on the sporophytic structures of the pistil and warrant further research.

The female gametophyte, wrapped within the ovular tissues, which are buried within the ovary, and, thus, more protected from the environment than its male counterpart, also

shows a shorter lifespan under temperature stress. Increasing the temperature from 5ºC to 25ºC in sweet and sour cherry (*Prunus cerasus* L.) reduced ovule longevity from 5 days to 1-2 days (Postweiler et al., 1985). Similarly, a constant temperature of 20ºC in controlled growth chamber reduced ovule longevity in plum (*Prunus domestica* L.) when compared to both field conditions and a lower constant temperature (Cerovic et al., 2000). A temperature above 25ºC induced degeneration and/or suppression of embryo sac development in peach (Kozai et al., 2004). Callose deposition at the ovule chalazal end has been traditionally used as a microscopic feature to assess early ovule degeneration (Vishnyakova, 1991). Callose deposits play different functions in plant development and, although the function of callose has been interpreted traditionally to isolate dying from living cells, callose might also inhibit sugar transport to the aborting embryo sac (Sun et al., 2004) inasmuch as it limited the diffusion of small sugar molecules to the developing muskmelon (*Cucumis melo* L.) embryo (Yim and Bradford, 1998). Whether this is a cause or a consequence of female gametophyte degeneration is not known. Likewise, the molecular mechanisms underlying ovule abortion under temperature stress are still unknown.

The responses of pollen, maternal sporophytic tissues and female gametophytes to temperature stress do not occur independently, but all three form integrated subsystems and interact actively during the progamic phase, from pollination to fertilization. In optimal conditions, the pistil is actively controlling pollen tube growth, and successful fertilization largely depends on the synchrony of pollen tube growth with both sporophytic tissue secretions and female gametophyte receptivity (Herrero, 2003). Furthermore, pollen tube growth in the pistil, or at least during its last growth stages within the ovary, seems to be guided by a diffusible signal produced by the synergids within the embryo sac (Higashiyama et al., 2001). Thus, any alterations on this intricate interaction pattern resulting in lack of developmental synchronization among the different subsystems or in either reduction in the carbohydrate availability for pollen tube growth or signaling molecules for pollen tube guidance might also lead to reduced fertilization. In this sense, work in torenia (*Torenia fournieri* Lind.) shows that pollen tubes lose, indeed, their directionality when the ovules have been exposed to high temperatures(Higashiyama et al., 2003). An integrative study of the whole process taking into account these complex interactions of the three individuals is lacking and requires further investigation.

In summary, temperature fluctuations negatively affect different stages of reproductive development and these effects largely explain erratic or low fruit and seed set in many plant species. Male gametophyte development is especially sensitive to temperature both at pre- and post-pollination levels. But the maternal tissues of the pistil and the female gametophyte, traditionally considered more tolerant, have also been reported to be

sensitive to temperature stress. Above these individual responses, temperature stress might lead to developmental asynchrony in pollen-pistil-ovule functioning leading to reduced fertilization levels. Even successful fertilization does not guarantee the expected seed and fruit set rates as long as persisting temperature stress halts embryo development (reviewed in Barnabás et al., 2008). But, as we will see in the next section, plant species do not respond equally to temperature stress, and reported works illustrate frequent instances of genotype-temperature interactions.

6. Genetic variation in the reproductive processes under temperature fluctuations

The fact that temperature stress during the flowering phase causes irregular cropping in many plants from different flowering plant families highlight the widespread sensitivity of the flowering phase to temperature fluctuations and suggests that some mechanisms may be conserved in temperature stress sensitivity. But there are several plant species that flower under extremely low or high temperatures, evidencing that some flowering plant species have developed strong strategies to cope with harsh environmental conditions. Thus, optimum temperature for some reproductive processes might reflect, indeed, species origin or adaptation. For example, while for many species native to or cultivated in temperate regions optimum temperatures in the range 15ºC-25ºC are usually recorded [examples include *Arabidopsis thaliana* (22ºC, Boavida and McCormick, 2007), sweet cherry (25ºC, Lewis, 1942), or almond (*Prunus dulcis* Mill.) and peach (16ºC and 23ºC respectively, Weinbaum et al., 1984)], optimum temperatures above 25ºC have been reported for several subtropical and tropical species such as *Datura stramonium* (33ºC, Buchholz and Blakeslee, 1927), *Oenothera* (33ºC, Lewis, 1942), groundnut (30ºC for pollen germination and 34ºC for pollen tube growth, Kakani et al., 2002) or cotton (*Gossypium hirsutum* L.) (from 28ºC to 32ºC, Kakani et al., 2005). These adaptations can be best seen in natural populations that flower during extremely high or low temperatures. Thus, the winter flowering species *Helleborus foetidus* and *H. bocconei* showed no decline in pollen viability even with minimum temperatures below -4ºC (Vesprini and Pacini, 2005). In Mediterranean regions, rosemary (*Rosmarinus officinalis* L.) flowers during the cool autumn and winter seasons while *Cistus incanus* L. and *Myrtus communis* L. flower during the warmest season of the year. When rosemary pollen was subjected during 4 hours to different temperatures, the best pollen viability was reported under low temperature (4ºC) and high relative humidity (100%) (Aronne et al., 2006), while *C. incanus* and *M. communis* displayed better pollen viability at low relative humidity and wide range of temperatures (Aronne, 1999).

Ottaviano and Mulcahy (1989) argued that due to pollen sensitivity to environmental conditions, pollen should show higher viability in the environment where the pollen

producing plant grows. This might lead to intra-specific variation in optimal temperature for reproductive development (Mckee and Richards, 1998). Intra-specific variability has indeed been extensively reported in cultivated plant species for many reproductive characteristics. Genotype-specific responses have been reported for stress applied during microsporogenesis affecting carbohydrate metabolism, degeneration of the tapetal tissues, pollen wall architecture, pollen morphology, pollen viability, anther dehiscence and pollen production (Aloni et al., 2001; Koti et al., 2005; Oliver et al., 2005; Porch and Jahn, 2001; Prasad et al., 2006; Pressman et al., 2002; Srinivasan et al., 1999; Suzuki et al., 2001). At the post-anthesis level, pollen germination (Hedhly et al., 2005a; Kakani et al., 2002, 2005), pollen tube growth rate (Clarke and Siddique, 2004; Hedhly et al., 2004; Srinivasan et al., 1999), and pollen dynamics (Hedhly et al., 2005a) also show genotype-specific responses.

Interestingly these variations in male gametophytic behavior seem to parallel male sporophytic characteristics (Clarke and Siddique, 2004; Hedhly et al., 2004; Kakani et al., 2002, 2005). Together with the large overlap in gene expression at the whole genome level between the two stages of the life cycle (Borges et al., 2008), selection at the gametophytic level can be effective and has been proposed as a complementary strategy to traditional sporophyte-based breeding strategies (reviewed in Hormaza and Herrero, 1996). Furthermore, gametophytic selection might have significant evolutionary implications as long as selection at the gametophytic level could change gene frequencies between generations (Hedhly et al., 2009).

Unfortunately, the genetic variation registered in cultivated plants is suffering erosion, by the introduction and monoculture of "improved" varieties. An effort to stop this loss of potentially interesting germplasm is of utmost urgency. The same conservation efforts should be applied to natural populations, where plant adaptation to environmental fluctuations depends largely on genetic diversity (Jump et al., 2009). Whether cultivated or not, species and genotypes that developed tolerance and/or avoidance strategies to temperature stress are, indeed, very interesting genetic reservoirs of potential temperature-tolerance genes. Their characterization might increase our understanding of how plants naturally cope with fluctuating environmental conditions. This will enable us to design target-oriented conservation strategies and efficient plant breeding programs under the current scenario of global climate change.

7. Perspectives

The sexual reproductive phase seems to be sensitive to temperature fluctuations in practically every stage depending on the intensity and extension of temperature stress and on the genetic material under study. Despite the abundant literature available on this subject, our understanding of temperature stress physiology during the reproductive

process is still incomplete and more research work is needed. Due to the complex interaction between different reproductive components targeted temperature stress to specific reproductive stages has been the most productive approach and more research work in different species will add more insights in the widespread sensitivity to temperature stress during plant reproductive development. But for many species, the sexual reproductive phase takes place during a short spell of time which has hitherto been a stumbling block to the appropriate targeting of temperature stress to specific developmental stages and this could explain at least some of the research works that report a negative effect of temperature stress during the reproductive phase. Fortunately, many woody perennial plant species predominantly hold a long sexual phase where developmental stages can be easily demarcated making them good experimental systems for studying in more depth the effect of temperature stress on particular stages. Another difficulty arises from the fact that during the sexual phase an active interaction takes place between genetically different subsystems (male gametophyte, female gametophyte and sporophytic tissues of the pistil) and to correlate an effect of temperature with an individual subsystem is a difficult task. Thanks to the fast progress in expression profiling and micromanipulation technologies, gene expression is being reported not only for individual structures such as the ovule, the anther, and the male gametophyte, but even at the single cell level such as egg cells and single sperm cells (reviewed in Borg et al., 2009). These approaches are good sound bases for surmounting these difficulties and studying stress response of a specific phase or component of sexual development.

Thermotolerance is a common response in plants to heat stress (reviewed in Ruelland and Zachowski, 2010; Wahid et al., 2007), and initial work in the sexual reproductive phase focused on Heat Shock Proteins (HSPs). Although they were first detected in reproductive tissues two decades ago (Vierling, 1991), their synthesis and function in these tissues are still controversial (Crone et al., 2001; Volkov et al., 2005), and the extent to which they mediate heat stress tolerance in reproductive organs needs to be clarified. Even in the widely characterized vegetative growth, HSPs are not the only players in plant thermotolerance, but additional multiple signaling pathways implicating other components, such as ethylene, abscisic acid, reactive oxygen species and auxin are playing an important role (Kotak et al., 2007; Sakata et al., 2010). A recent expression profiling analysis of maturing tomato microspores subjected to a short heat shock treatment reveals an up-regulation not only of genes implicated in similar pathways, but also of some pollen specific genes that might represent pollen-specific response mechanisms (Frank et al., 2009). The effect of high temperature and high light induced different transcriptome responses between vegetative and reproductive tissues of sunflower with some specific functions in both tissues (Hewezi et al., 2008). Likewise,

work in Arabidopsis shows that gene regulation underlying cold acclimation in vegetative tissues does not trigger expression of the same genes in pollen undergoing cold stress (reviewed Chinnusamy et al., 2008). All these results indicate that, although the sexual reproductive phase shares some common mechanisms of temperature stress response with vegetative growth, it seems to have also its own physiology. Thus, future progress in expression profiling and functional genomics targeted to this phase will help to unravel the genetic basis of temperature tolerance during sexual development in plants.

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FIGURE AND TABLE LEGENDS

Figure 1

Effect of temperature stress on the sexual reproductive phase. Studies showing negative effects of temperature stress on fruit and seed are reported to be due to temperature interference with the development of sexual organs before pollination, with their function during the progamic phase (from pollination to fertilization), and with early embryo development. Interference at the pre-pollination level spans from complete male and female sterility and flower abortion to reduction in the quantity and quality of the pollen produced. Poor functioning at the post-pollination level resulting in altered fertilization of male and female structures developed under temperature stress is also reported. Temperature stress confined to the progamic phase shows negative effects on each subsystem (male gametophyte, female gametophyte and sporophytic tissues of the pistil) as well as on their interaction; all effects alter the fertilization level. Although scarcely studied, early embryo development has also been shown to be sensitive to temperature stress. Reports showing these effects on many flowering plants are detailed in Supplementary table 1. The drawing in the middle panel represent a pistil with its three typical organs: stigma, style and ovary; a pollen grain (red) with its pollen tube (yellow) that have reached the embryo sac are drawn . The drawing on the lower panel represent a young embryo (red) growing within a fertilized ovule

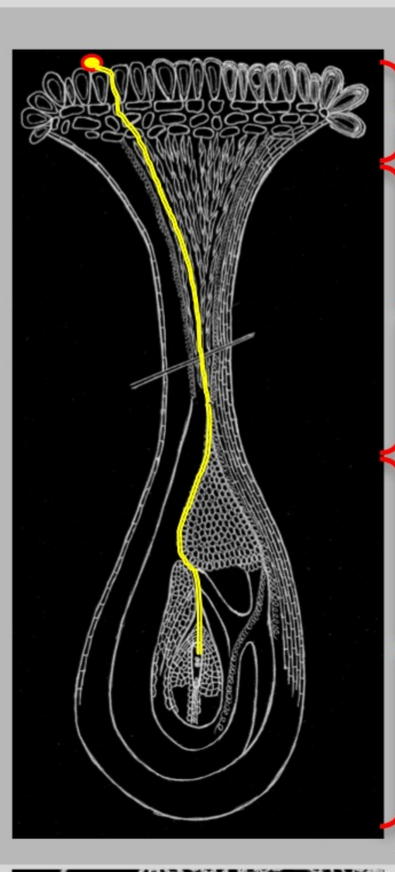
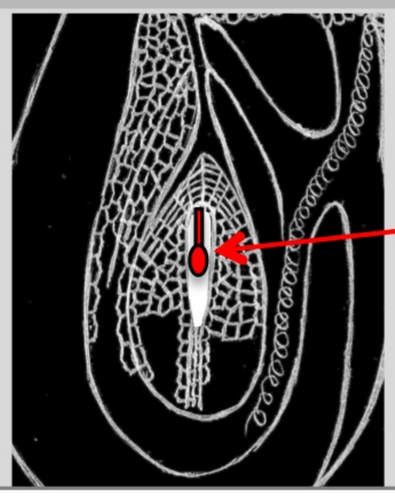
Supplementary table 1

Selected references from tropical, subtropical and temperate fruit tree species, from cultivated herbaceous flowering species, and from wild and model flowering plant species showing negative effects of temperature fluctuation during the flowering phase on fruit and seed set. The reported sensitive repoductive stages and the underlying mechanisms are given.

Research Highlights

- Small variations in temperature hinder sexual processes and affect seed set
- Temperatures with no effects on vegetative growth might alter sexual development
- Temperature stress affects male-female interaction besides individual effects
- Plants species do not respond equally to temperature stress
- Many instances of inter- and intra-specific genetic variations are reported

Figure 1

Affected developmental processes			Reproductive effects
Sexual organ development	Androecium development	Microsporogenesis Microgametogenesis Anther development	Reduction in pollen quantity and viability Male sterility Flower abortion Poor post-pollination functioning of pollen grains/pollen tubes
	Gynoecium development	macrosporogenesis macrogametogenesis Pistil development	Female gametophyte absent or degenerated Malformed style and/or ovary Poor post-pollination functioning of pistil structures and ovule
Pollination			
Progamic phase		<u>Stigma</u> Pollen germination Stigma receptivity <u>Style</u> Pollen tube growth Style receptivity <u>Ovary</u> Pollen tube growth Ovule receptivity	Poor pollen adhesion and germination Altered stigmatic receptivity duration Altered pollen tube growth rate Pollen tubes population census affected Pollen tube guidance disturbed Ovule viability and longevity affected Asynchronous pollen-pistil interaction
Fertilization			
Embryo development		Embryo Development	Early embryo abortion

Supplementary table 1 Selected references from tropical, subtropical and temperate fruit tree species, from cultivated herbaceous flowering plant species, and from wild and model flowering plant species showing negative effects of temperature fluctuation during the flowering phase on fruit and seed set. The reported sensitive repoductive stages and the underlying mechanisms are given.

Species	Type of stress	Stage of stress application	Effects on fruit/seed set	Sensitive stage	Underlying mechanism	References
Tropical, subtropical and temperate fruit tree species						
Annona cherimola	Cool 20/15ºC[(1)]. Warm 30/25ºC (in both cases 12h day temperature) RH 50-80%, light 10-14h.	During two consecutive years. Reciprocal pollinations combining warm/cool developed pollen/pistil.	Substantial reduction in fruit and seed set and seed number per fruit. Slow fruit development and some of them malformed.	Both pollen and pistil development affected at pre- and post-anthesis.	Pollen viability and germination, stigmatic receptivity. Pollen was more sensitive.	Higuchi et al., 1998
Mangifera indica	Low temperature: 20/10ºC. Optimum temperature: 25/15ºC.	Low temperature treatment starting 2-5 days after pollination and lasting for 3 days in all cases	Increase in percentage of nubbins (genotype-specific response).	The major effect occurred when stress applied 3 days after pollination.	Early embryo abortion.	Sukhvibul et al., 2005
Olea europea	20ºC, 25ºC and 30ºC constant temperatures.	Just before blooming, hand cross-pollination.	Fruit set inhibited at 30ºC (all pistils abscised by two weeks after bloom), best fruit set at 25ºC.	Fertilization and/or early embryo development at 30ºC. Pollen tube growth rate at 20ºC.	Enough entry of pollen tubes to ovules (47% at 30ºC) but no growth of functional ovules and ovaries. Delayed and reduced fertilization at 20ºC due to slower pollen tube growth.	Cuevas et al., 1994
Persea americana	17/12ºC, 25/20ºC, 33/25ºC.	Starting at first flower bud bursting.	Lower fruit set at high and low temperature for 'Fuerte' genotype.	Flower development and the progamic phase.	Abscission of unopened flowers at 33ºC and no fertilization at 17ºC.	Sedgley, 1977
	17/12ºC, 25/20ºC, 33/25ºC.	Starting at first flower bud bursting.	Lower fruit set at high and low temperature; more reduction at the higher temperature.	Embryo development.	Higher fruitlet abscission at 33ºC and slower embryo growth at 17ºC. 'Hass' is more consistent than 'Fuerte'.	Sedgley and Annels, 1981
Prunus armeniaca	METS in field conditions, 2.7-3ºC average increase	Two weeks before anthesis (starting when bud scales separate and sepals protrude).	Reduction in fruit set from 36% to 21% one year and 49% to 32% another year	Pistil development at pre-anthesis (increase in the proportion of abnormal flower with short style and non-swollen ovaries)	Might be due to flower quality. Asynchronous development of pistils (underdeveloped) in relation to premature flower opening.	Rodrigo and Herrero, 2002
Prunus avium	METS in field conditions, 3ºC average increase.	2 weeks from pollination.	Reduction from 18% to 5% in one year and from 25% to 4% in another year.	Progamic phase.	Stigmatic receptivity and pollen tube growth in the ovary.	Hedhly et al., 2003, 2007
Prunus domestica	Field conditions in 6 orchards. One cold spring (7-10ºC) and one warm spring (14-17ºC).	Temperature measured during 3 weeks following full bloom.	Fruit set ranged from 36 to 64% in the warm spring and from 1-13% in the cold spring.	Embryo sac longevity and pollen tube growth.	Cool temperature slow down pollen tube growth so long that ovules begin to degenerate.	Thompson and Liu, 1973

Prunus persica	Constant temperature of 15ºC, 20ºC, 25ºC, 30ºC, and natural conditions (average 13.5ºC).	Starting one month before blooming, two months later all trees transferred to the field.	Scarce fruit set above 25ºC (less than 3% compared with 30% in natural conditions, 35% at 15ºC.	Embryo sac development.	Degeneration or suppression of embryo sac development above 25ºC, pollen germinates and elongates normally.	Kozai et al., 2004
Cultivated herbaceous species						
Arachis Hypogaea	Control 28/22ºC. Heat stress: different temperatures from 28ºC to 48ºC.	28/22ºC from sowing to 9 days after flowering, then different temperatures varying time of the day and duration of exposure.	Reduction in the number of pegs or pods per plant with increasing temperature.	Flower and seed development.	The effect of temperature varied with the duration of exposure and time of the day.	Prasad et al., 2000
	32/22ºC, 36/26ºC, 40/30ºC, and 44/34ºC. Ambient and doubled CO2 concentration.	Season long exposure from emergence to maturity.	Reduction in seed yield with increasing temperature, smaller seed size, fewer seeds per pod and decreased shelling.	The reproductive phase is more sensitive to inhibition by super-optimal temperature than vegetative growth.	Despite the increase in vegetative growth pollen viability has been adversely affected leading to lower fertilization. Seed filling was also affected.	Prasad et al., 2003
Brassica napus	Control 23/18ºC 8/16h dark/light. Heat stress: day temperature of 23ºC ramped at 2°C/h over 6 h, to 35°C, maintained at 35ºC for 4 h, and then ramped back down to 23°C at 2°C/h back over 6 h.	1 or two weeks since initiation of flowering.	Flowering not affected but fruit, seed development and weight significantly reduced. Flower-to-silique development reduced by 35% and 43% after 1 and 2 weeks stress respectively.	Male and female gametophyte function.	No effect during microsporogenesis. Pollen germination rather than viability affected. Pollen-to-ovule ratio affected. Greatest effect when both gametophytes stressed.	Young et al., 2004
Capsicum annum	1) 6, 48 and 120h at 33ºC, control is 25/21ºC. 2) 33ºC for 3 days before anthesis or 2 days after anthesis.	1) Heat stress from pre-microspore mother cell to maturing microspores, anthesis and pollination. 2) Heat stress just prior anthesis or just after.	Significant reduction in fruit set, fruit size and seed number.	Meiosis and post-pollination function.	Inhibition of pollen development from tetrad formation to dissolution. Stress just prior anthesis had no effect on pollen and pistil. Inhibition of fertilization and early fruit development.	Erickson and Markhart, 2002
var. Shishito	Control: 30/22ºC. Heat stress: 38/30ºC.	Different extensions: 1- Anthesis (A) – 5 days after anthesis (DAA) 2- A – 10 DAA 3- 10 DAA – 30 DAA 4- 30 DAA - Harvest 5- A – Harvest.	Parameters significantly affected: Seed set, fruit size and weight, fruit growth period and seed quality.	Seed set was mostly affected from anthesis to 10 DAA. Reduction in seed set affected fruit size and weight.	Not studied but A – 5 DAA seems to affect fertilization (non abnormal seeds), and A-10 DAA seems to affect early embryo development (high proportion of malformed seeds). Later stresses affected fruit size, seed quality and fruit growth period.	Pagamas and Nawata, 2008
Licopersicon esculentum	Controls: 26/22ºC and 28/22ºC. Moderately elevated temperature stress (METS): 32/26ºC.	Starting at 41 days after sowing. One treatment consisted of a relief from high temperature 10 days before anthesis.	One genotype out of five set fruits under high temperature.	Pollen development and germination.	The number of pollen grains produced was not affected. No relationship between effect on photosynthesis, on night respiration and on the number of pollen grain produced and fruit set.	Sato et.al., 2000

	Controls: 28/22ºC. METS: 32/26ºC.	Initial growth at both temperatures and then different treatments of relief from high temperature before and after anthesis.	Fruit set inhibited unless a relief of more than five days before anthesis.	The most sensitive period spans over two weeks before anthesis.	Disruption of pollen development, irregularities in endothecium, epidermis and stomium.	Sato et al., 2002
	Control: 28/22ºC. METS: 32/26ºC.	Two months after sowing.	Reduction in the number of fruit set.	Pollen development (Reduction in pollen viability and the number released).	Disruption of sugar metabolism (increase of sucrose and decrease of acid invertase) and proline translocation.	Sato et al., 2006
Linum usitatissimum	Control: 23/18ºC, 16/8h light/dark. Heat stress: During light temperature raised from 18ºC to 40ºC at 3ºC/h over 7 h, held 2h at 40ºC, and returned over 7h to the 18ºC dark temperature.	7 or 14 days heat stress applied 12 days after first flower opening	Flower production not affected; significant reduction in boll formation and seed set.	Male and female gametophyte function.	Pollen tube growth in the ovary.	Cross et al., 2003
Oryza sativa	Heat stress: 37.5/26ºC using sun-lit phytotrons.	Before anthesis (middle heading stage), 6h (10:00 – 16:00) of heat stress for 6 consecutive days.	Reduced fertility (seed set).	Anther dehiscence varied between genotypes having different anther cell layers.	High correlation between fertility and the number of cell layers between the locule and and the lacuna formed between septum and stomium.	Matsui and Omasa, 2002
	Control: 28/22ºC Heat stress: 33/27ºC using Temperature Gradient Greenhouses.	From emergence to harvest during two consecutive seasons.	Reduced spikelet fertility (seed set), which lowered grain yield, which in turn affected the Harvest Index. Greater effect on yield than on biomass.	Late stages of pollen development, anthesis and probably the progamic phase. Genotype-dependent effect.	Reduced pollen production and anther dehiscence. The latter effect is behind poor pollen shed and the number of pollen grain on the stigma	Prasad et al., 2006
	Control: 28/22ºC. Heat stress: 39/30ºC.	Experiment 1: heat stress applied 7 days at 3 stages of panicle development Experiment 2: heat stress applied during 2, 3 and 4 days at early microspore stage	Experiment 1: fertility totally lost when stress coincided with early microspore Experiment 2: reduced fertility paralleling exposure duration	Early microspore development and pollen adhesion and germination at the stigma level	Reduced pollen adhesion and germination could be explained by an earlier effect on tapetum (repressed genes without degeneration) that would have affected pollen development and maturation.	Endo et al., 2009
Phaseolus vulgaris	Control: 22/17ºC. Heat stress: 32/27ºC.	Heat stress applied during few days at micro and macro-sporogenesis, at pollen and embryo development and at anthesis and early pod development.	Lower pod and seed set Lower seed set at distal position.	Microsporogenesis and gynoecium function.	At sporogenesis pollen viability and anther dehiscence. At anthesis pollen tube growth and fertilization.	Gross and Kigel, 1994
	1: Control: 27/23ºC 12/12h light/dark, 70% RH.; Heat stress: 32/28ºC. 2: different sowing dates (ensured different field temperature regimes) of different cultivars	1- Heat stress during 24h (09:00h-09:00) in growth chamber; plant transferred back to greenhouse	1- Pod set reduced for flowers opening 1 or 8 to 11 days after heat stress application. 2- Pod set reduced (even null) for sensitive cultivars when flowered at high field temperature.	Early microspore development and gynecium function	High correalation pollen viability-pod set 8 to 11 days before anthesis reveal that early microspore development is highly sensitive. But the anthesis period appears even more sensitive.	Suzuki et al., 2001

	28/18ºC, 31/21ºC, 34/24ºC, 37/27ºC and 40/30ºC. Ambient and doubled CO2 concentration.	From emergence to maturity	Increase in vegetative development, but reduction in pod-set, seed number, and seed-set for temperatures above 34/24ºC.	The beneficial effect of CO2 during vegetative growth did not offset temperature stress negative effect on pollen development.	Pollen production and pollen viability.	Prasad et al., 2002
Triticum aestivum	Control: 20°C Heat stress: 30ºC during 3 days.	At the onset of meiosis in the anther.	Reduction in seed set by 21%	Embryo sac and nucellus development	Reduced pollen tube growth in the ovary, callose deposition in pollen tubes and reduced fertilization.	Saini et al., 1983
	Control: 20°C Heat stress: 30ºC during 3 days.	During meiosis in pollen mother cells.	Not checked but they reported from complete to partial male sterility.	Young microspores and pollen grain mitosis 1.	Premature tapetal degeneration and loss of contact between microspores and tapetum.	Saini et al., 1984
Vigna unguiculata[3]	Optimum night temperature: 33/20ºC. High night temperature: 33/30ºC. 45-55% and 65-75% day/night RH. Photoperiod 14-15h.	1- Stress applied during the whole cycle. 2- Plants were reciprocally transferred between chambers at different development stages and during 6 days.	No pod set under high night temperature. This negative effect was a developmental-specific response	Pollen viability and anther dehiscence (the greatest sensitivity at 9 to 7 days before anthesis).	Premature degeneration of the tapetal layer after microspore release from the anther, and lack of development of the endothecial layer.	Ahmed et al., 1992
	33/20ºC versus 33/30ºC in combination with 350 and 750 umol.mol^{-1} of CO_2 45-55% and 65-75% day/night RH. Photoperiod 14h.	During the whole cycle.	Under high night temperature with either ambient or elevated CO_2, heat-sensitive lines produced no flowers and/or no pods, whereas the heat-tolerant line abundantly set pods.	Floral buds and anthers in sensitive lines	CO2 increases carbohydrates in sensitive lines (starch in leaves, stems and peduncles), but low sugar level was found in peduncles. Thus, heat seems to have greater effects on assimilate demand than on leaf assimilate supply.	Ahmed et al., 1993
Wild and model species						
Arabidopsis thaliana	Control: 22ºC/60% RH. Heat shock: 42ºC/85% RH.	4 hours when first flower was at stage 12[2] (petal visible and enclosed within sepals) in 3 weeks old plants.	Short and tiny siliques for some flower positions within the inflorescence.	Pollen meiosis and anther dehiscence.	No pollen produced when heat shock coincided with meiosis (flower stage 9). At flower stage 12, sterility was due to failure in pollen release rather than to pollen viability.	Kim et al., 2001
	Control: 20ºC/65% RH Heat Stress: 32, 34, 36, 38, or 40ºC varying humidity to maintain the same vapor pressure deficit.	At first flower opening, treatment applied during 24h, 48h, 72h, 96h and 120h.	Production of short and empty siliques at 32 ºC and 34ºC. Higher temperature induced bud abortion, inflorescence abortion or even plant death.	Sensitive stage varied with ecotypes; Col-0 aborted buds at early flower stage 12, and No-0 aborted buds at approximate stages 9-12.	Pollen sterility suggested as the cause of short and empty siliques. Aborted buds corresponded to post meiosis late pre-anthesis in col-0 and from meiosis to late pre-anthesis in No-0.	Warner and Erwin, 2005

	Control: 20ºC/18ºC, 16/8h light/dark. Hot-cold stress: 40ºC/-1ºC, 16/8h light/dark.	Hot-cold stress from initiation of bolting to silique maturity. At daybreak gradual shifting during 5 h from -1ºC to 40ºC, keep 1 h at 40ºC followed by a drop to 10ºC for 10 h. At onset of night, drop temperature to -1º for 8 hours.	Decreased seed set in an ecotype-specific manner. In the sensitive ecotype seeds were clustered in the top half of the silique.	Negative effects on pollen tube growth and guidance.	The registered effect depended on the ecotype, and an effect on the transmitting tract or ovule receptivity cannot be ruled out.	Zinn et al., 2010
	Control: 23ºC constant temperature, 16/8h light/dark. Heat stress: 30ºC, 31ºC or 33ºC.	Stress applied during anther development of the primary inflorescence between 1 and 11 days	No specific data on seed set but pollen sterility indirectly would reduce seed set in this species	Some heat stress/duration combination hampered stamen development (microsporogenesis, filament elongation and pollen maturation)	Endogenous auxin synthesis seems to be hampered in anther wall and microspores and could explain sterility as exogenous auxin restored sterility	Sakata et al., 2010
Primula sp	6ºC, 15ºC, and 26ºC, 15h/day.	Seven populations of five species subjected to 3 different temperatures during 4 days after pollination and then returned to 15ºC.	Temperature affected capsule and seed set, seed number, weight and physiology in a species-specific way. 26ºC was detrimental for most species.	Progamic phase.	Pollen germination and pollen tube growth were affected; female attributes not evaluated.	Mckee and Richards, 1998

(1) 20/15ºC here and so forth stand for day/night temperature regimes.
(2) As described by Smyth et al., 1990.
(3) For more references on the extensive work done in this species, and other earlier publications in other species please refer to Hall (1992)

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